Journal of Alzheimer's Disease Reports 8 (2024) 627–635
DOI 10.3233/ADR-240012
IOS Press

Review

Circulating Cell Free DNA and DNA Double-Strand Breakage in Alzheimer's Disease

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Received 21 January 2024
Accepted 5 March 2024
Published 8 April 2024

Abstract. Alzheimer's disease (AD) is an age-related neurodegenerative disease that is characterized by memory loss and multiple cognitive impairments. AD is pathologically characterized by age-dependent accumulation of amyloid-β protein and the phosphorylation of tau protein in the brains of patients with AD. Clinically, manifestations of AD include cognitive decline, dementia, alterations of high-order brain functions, and movement disorders. Double-stranded DNA breaks are a lethal form of DNA damage and are typically repaired via non-homologous end joining and homologous recombination. However, in AD brain, repair mechanism is disrupted, leading to a cascade of events, cognitive dysfunction, organ failure and reduced lifespan. Increased circulating cell-free DNA in the blood, cerebrospinal fluid, and urine in patients with AD, can be used as early detectable biomarkers for AD. The purpose of our article is to explore the potential uses of cell-free DNA and double-stranded DNA breaks as prognostic markers for AD and examine the recent research on the application of these markers in studies.

Keywords: Alzheimer's disease, cell-free DNA, dementia, double-stranded DNA breaks

INTRODUCTION

The number of Americans older than 65 years is expected to double, from 40.2 million in 2010 to 88.5 million in 2050 [1]. This older cohort is more susceptible to disease and has a higher mortality probability. Age emerges as a principal risk factor for a range of diseases, including neurodegeneration, cardiovascular disorders, diabetes, osteoporosis, and cancer [2]. With this increase in the elderly population and the prevalence of disease, understanding the physiological process of aging, pathological

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disorders associated with aging, and the need for preventative and therapeutic interventions become increasingly important. Aging is characterized by a progressive deterioration at the molecular, cellular, and tissue levels. Numerous processes and factors have been identified as central to the biological aging mechanism, including genomic instability, telomere attrition, epigenetic changes, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication, with genomic instability as a primary driver [3]. Individuals also experience structural changes with increasing age. There is often a prominent deterioration of the gray matter volume after the age of 20 years, especially in the prefrontal cortex [4]. Decreases in the temporal lobes and volume of the hippocampus also occur [5]. The possible causes of a decline in gray matter volume include the death of neurons, a decrease in the size of neurons and number of connections between them, and beta-amyloid protein accumulation in the brain, which is present in cognitively normal patients but is often an important predictor for the progression to Alzheimer's disease (AD) [4, 6, 7]. The decline in white matter volume is typically greater than gray matter volume [8].

AD is an age-related neurodegenerative disease that progresses over time and causes cognitive and memory impairment [9]. One in ten individuals at age of 65 or older has AD, alluding to the vast economic, emotional, and physical impact of the disease on the elderly population [10]. AD is pathologically characterized by amyloid-β protein (Aβ) accumulation, plaque formation, neurofibrillary tangles, and the hyperphosphorylation and polyubiquitination of the tau protein [11, 12]. Clinically, manifestations of AD include cognitive decline, dementia, alterations of high-order brain functions, and movement disorders [13].

The increased prevalence of AD emphasizes the importance of using accurate measures or predictors of the onset of disease. However, there is currently no gold standard tool for assessing healthy aging and no single biomarker has been identified as a highly sensitive and specific measure [14]. Amyloid positron emission tomography (PET), cerebrospinal fluid (CSF) concentrations of amyloid and tau proteins, and plasma concentrations of amyloid, tau, and protein are classified as pathophysiologic biomarkers. Neuroimaging, such as tau PET, fluorodeoxyglucose PET, and structural magnetic resonance imaging, have also been used as topographic biomarkers [14]. Blood-based biomarkers have emerged as another option for early diagnosis. Aβ peptides and phosphorylation of tau (pTau) in the blood have been associated with their corresponding concentrations in CSF. Measures of plasma $A\beta_{1\text{-}42}$, t-Tau, p-Tau_{181}, p-Tau_{217}, p-Tau_{231}, NfL, and apolipoprotein E have shown promise in identifying AD pathology [14, 15].

Recent research revealed that increased double-stranded DNA breaks (DSBs) in the brain and accumulation of circulating cell-free DNA (cfDNA) in the blood and CSF are of great importance because of their utility as peripheral early detectable biomarkers of AD.

Circulating cfDNA is a prospective biomarker found in blood, CSF, and urine and can be used to measure nuclear or mitochondrial DNA (mtDNA) that has been released following apoptosis or necrosis [16]. cfDNA has been associated with mortality and frailty and is potentially released in those contexts due to increased cellular senescence and catabolic processes [17]. One study by Nidadavolu et al. found that elevated cfDNA levels were associated with a higher risk of dementia as well as more rapid decline in cognitive and physical functioning, thus bringing attention to cfDNA for use as a marker of higher risk of dementia, poor cognition, and frailty [16].

Unrepaired breaks in DNA strands have also been implicated in causing neurological disorders. DSBs are particularly damaging and can lead to cell death if left unrepaired or incorrectly repaired. Individuals with genetic mutations that impair DNA repair pathways have demonstrated accelerated neuronal death, which can manifest as neurodegeneration [18]. Many studies have also linked aging and decline in DNA repair activity [19–21]. DSBs also have a potential to act as a prognostic biomarker in patients with neurodegenerative disorders. The comet assay, also known as alkaline single-cell gel electrophoresis, can be used to visualize and measure DSBs in individual cells [22].

This paper will further explore the potential uses of cfDNA and DSBs as prognostic markers for AD and examine the recent research on the application of these markers in studies.

CIRCULATING CELL-FREE DNA AND ITS RELATIONSHIP TO AGE-RELATED DECLINE

cfDNA mostly consists of double-stranded nuclear DNA and mtDNA, and it is suggested that the major-

ity of cfDNAs in both healthy and ill patients are released during the process of apoptosis [23]. Elevated cfDNA concentrations have been observed in various conditions, including cancer, pregnancy, post-transplantation, and in disorders such as inflammation, diabetes, and myocardial infarction [24, 25]. The concentration of cfDNA in blood varies between 0–5 and > 1000 ng/ml in patients with cancer and between 0 and 100 ng/ml in healthy subjects. It can also vary according to different tumor types [26]. Levels of cfDNA have also been reported to be increased in older individuals, potentially due to the stressful effects of aging on cells, such as increased DNA release, inflammation, and apoptosis [27].

There are multiple proposed mechanisms of cfDNA release into circulation. Apoptosis has been considered to be the primary source of circulating cfDNA [28]. Apoptosis is a process that is essential to homeostasis and the turnover of cells. Intrinsic factors, such as oxidative stress and DNA damage, and extrinsic factors, like receptor-ligand interactions, can instigate apoptosis [29]. In the context of AD and other neurodegenerative disease, post-mitotic cells of the nervous system are usually resistant to apoptosis, but apoptosis may become upregulated in the pathogenesis of these diseases [30]. Necrosis also contributes larger fragments to the pool of cfDNA. Erythroblast enucleation is also another potential source of cfDNA [31].

Another significant mechanism is the process of NETosis. In NETosis, activated neutrophils release nuclear DNA in response to stimuli, resulting in the formation of neutrophil extracellular traps (NETs), which consist of proteins with antimicrobial properties [32]. Active DNA release serves to eliminate damaged DNA, thereby maintaining cellular homeostasis and averting inappropriate immune responses [32]. The secretion of DNA can also have pathological effects, such as promoting inflammation. Histones, for example, can trigger a proinflammatory response via toll-like receptors, resulting in the production of cytokines, and induce NET formation as well as thrombosis and renal dysfunction [33]. mtDNA is also implicated in promoting inflammation [34]. Another example of a pathological effect of DNA release is how NETosis has been indicated in influencing cancer microenvironments, promoting tumor growth, and facilitating cancer metastasis [35].

Levels of extracellular DNA in circulation are balanced by the processes of DNA release and DNA clearance. cfDNA clearance can occur in their tissue of origin, blood, or other bodily fluids or organs, such as the liver, spleen, kidney, or lymph nodes [36]. In the healthy individual, cfDNA clearance is optimal and the levels of circulatory cfDNA are low due to the rapid clearance of apoptotic cells and cfDNA. However, in the cases of malignancies, chronic inflammation or excessive cell death, cfDNA can accumulate due to insufficient clearance [37]. Therefore, the correlation of high levels of cfDNA and pathological conditions could be explained by ineffective cfDNA clearance mechanisms.

The levels of total cfDNA, unmethylated cfDNA, RNase P-coding cfDNA and Alu repeat have been found to differ between old and young controls [27]. The explanation for these variations could be attributed to increased cellular senescence and death and decreased clearance that occurs with increased age. The same study found that higher total cfDNA levels and unmethylated cfDNA were associated with frailty, lower body strength, cognitive impairment, decreased mobility and challenges with daily functioning [38]. In another study, high levels of cfDNA were associated with lower global cognitive function, and there was a dose-response relationship between increasing levels of cfDNA and the odds of mild cognitive impairment (MCI) and dementia [39].

CLINICAL USES OF CFDNA AND RECENT APPLICATIONS TO AD

Liquid biopsy offers an alternative to solid tissue biopsy by sampling body fluids and analyzing molecular components released from cells. Fluids include plasma, serum, CSF, urine, saliva, pleural effusions, and more [40–42]. This approach is less invasive and offers a more holistic view of disease compared to tissue biopsy [43–45]. Liquid biopsies can sample numerous genetic markers from free nucleic acids, including cfDNA, cell-free mtDNA, circulating tumor DNA, and cell-free RNA, extracellular vesicles, proteins, and metabolites [46].

Despite the advantages and exciting potential of liquid biopsy, there are challenges that limit its diagnostic potential. Circulating tumor DNA for instance is released from tumors in minimal amounts in early-stage disease, often below the level necessary for detection [47]. Sample standardization and cost effectiveness are additional challenges facing the widespread adoption of this technique [48–50]. Finally, confounding factors, comorbidities, risk factors, and reproducibility must be considered and minimized for clinical application.

However, DNA methylation can provide the key to improving the specificity of cfDNA. DNA methylation, which takes place on CpG islands composed of cytosine-guanine dinucleotide repeats, is a process that can prevent gene transcription in the context of a promoter region [51]. DNA methylation in the promoter region can in turn regulate chromatin configuration, which has been associated with gene silencing [52]. Different cell types and tissues experience varying amounts of genomic methylation. These differentially methylated regions (DMRs) serve as a unique marker of cell type [53]. Using a catalog of cell type-specific methylation patterns, the specificity of cfDNA as a biomarker can improve by allowing the identification of specific tissues of origin. In one study, methylome analysis of cfDNA demonstrated that for certain solid cancers, such as colon, breast and liver cancer, the tumor tissue of origin made up the majority of the total cfDNA pool [53]. In pediatric patients, the use of cfDNA methylation deconvolution resulted in the correct identification of 81% of samples from patients with extracranial tumors [54]. Methylation analysis with cfDNA can provide insight into the patient's response to treatment, tracking decreases in tumor cfDNA in response to therapy, identification of the primary tumor type, and determining the prognosis and risk of the patient [55]. The utility of methylated analysis and cfDNA in determining the tissue origin of tissue injuries can potentially be applied to neurons and other brain cell types, offering another avenue for the use of cfDNA as a biomarker for neurodegenerative diseases.

Brain somatic mutations have been proposed to contribute to the development of neurodegenerative diseases [56]. In one study, researchers looked at somatic mutations in the hippocampal formation of AD patients and demonstrated that brain somatic mutations that accumulate with age can also be involved with the appearance of tau pathology in the hippocampal formation of AD brains [57]. In a study by Ye et al. (2021), it is proposed that CSF liquid biopsies with cfDNA can also be used to detect somatic mosaicism in non-malignant brain diseases, specifically epilepsy. The study showed that there is adequate cfDNA in CSF to utilize for liquid biopsy and higher levels of cfDNA concentration were observed in epilepsy patients in comparison to controls [58]. While the study focused on the use of cfDNA in tracing somatic mutations in the context of epilepsy, it does open the possibility of utilizing a similar methodology for detecting and tracking the progression of neurodegenerative diseases like AD.

In a study by Pollard et al. (2023), several key steps were performed involving blood plasma samples obtained from various cohorts, including AD patients, individuals with MCI, purified cortical neurons, and healthy controls. Publicly available data were analyzed to identify DMRs between neurons and blood plasma samples. This allowed researchers to pinpoint specific genomic regions with distinct methylation patterns, potentially indicative of neuron-derived cfDNA. Primers were designed to target the identified DMRs, and cfDNA was extracted from blood plasma samples. Bisulfite conversion and PCR amplification were performed to prepare the samples for sequencing analysis. Sequencing data obtained using nanopore technology were analyzed to assess methylation patterns at the target loci. Whole-molecule analysis allowed for precise identification of neuron-derived cfDNA, distinguishing it from other sources of cfDNA in the bloodstream. Results demonstrated significant differences in methylation patterns between neuron-derived cfDNA and blood plasma cfDNA, particularly at specific genomic loci. Elevated levels of neuron-derived cfDNA were consistently observed in AD patients, aligning with the expected pathology of neurodegeneration associated with the disease. This observation suggests that the proposed diagnostic approach has the potential to effectively identify individuals with AD, even in the absence of clinical symptoms. Additionally, this study provided insights into the prognostic utility of neuron-derived cfDNA levels in individuals with MCI. Remarkably, all MCI patients who later progressed to AD exhibited elevated levels of neuron-derived cfDNA at the time of sample collection. This finding underscores the predictive value of the proposed biomarker, offering the possibility of early intervention and proactive management strategies for individuals at risk of developing AD [59].

Podleysniy et al. (2016) aimed to differentiate between AD and rapid progressive dementia by assessing circulating cell-free mtDNA levels in CSF and investigating the impact of neuronal damage on CSF mtDNA content. The study revealed that a drop in CSF mtDNA concentration exceeding 50% distinguished AD from confirmed sporadic Creutzfeldt-Jakob disease and other neurologic disorders or dementias. This finding aligns with previous research indicating that low-CSF mtDNA distinguishes preclinical or clinical AD from frontotemporal dementia. Overall, the study suggests that low CSF mtDNA content is not a general marker of neurodegeneration and its specificity allows for

the differentiation of AD from sporadic Creutzfeldt-Jakob disease and other dementias [60].

DSBS AND AD

DSBs are a lethal form of DNA damage and are typically repaired via non-homologous end joining and homologous recombination. Non-homologous end joining (NHEJ) is more error-prone and less precise in comparison to homologous recombination and is the primary pathway of DSB repair in neurons [61]. As people age, small errors can occur in these repair systems and accumulate to the point of causing genomic instability, resulting in cellular dysfunction or death [62]. It has been shown that a loss of NHEJ activity in adults can lead to neurodegenerative diseases [63]. In addition, faulty NHEJ has been found to accelerate aging in mice [64].

DNA-dependent protein kinase is a multi-subunit enzyme that is essential to non-homologous end joining as it initiates the DNA repair process [61]. Ku70/Ku80 is a heterodimer regulator of DNA-PK and acts as an activator [65]. Mice with defective Ku80 were found to have impaired NHEJ, poor telomere maintenance, and premature aging, although no Ku80 deficiency has been reported in humans [64]. In another study, Ku-DNA binding was reduced in extracts of post-mortem AD mid-frontal cortex, which could be indicative of reduced levels of Ku and DNA-PKcs level, which is a subunit of the DNA-PK holoenzyme [66]. In addition, a later study showed that NHEJ and DNA-PKcs levels were reduced in extracts from brains of AD versus normal subjects [67]. While AD is associated with NHEJ impairment and lower levels of DNA-PKcs, it is uncertain if reduced DNA-PK is a primary cause for impaired NHEJ or if NHEJ already declines in AD and secondarily results in reduced DNA-PK activity.

AD is also associated with an impaired DNA end-joining activity, leading to susceptibility to damage, such as DSBs [68]. DSBs accumulated in AD brain cells were reported [69]. In one study, using antibodies against γH2AX, a biomarker for DSBs, it was found that AD brains contained 18 times more DSBs than control brains and the pattern of distribution of DSBs also differed between the two groups [70].

Although DNA damage has been associated with neurodegenerative diseases, it is unclear whether increased DNA damage is a consequence of or the primary cause of disease progression. Some studies suggest that elevated DSB accumulation or decreased DNA repair proteins occur in the brains of AD patients and can precede tangle formation [71].

Depletion of BRCA1 in the hippocampus of mice elevates DSBs, reduces the size of neurons, alters the functioning of neurons, increases neuronal excitability, and impairs learning and memory [72]. Neuronal death as a response to DSBs often involves processes like ATM activation and reactive oxygen species generation [72]. The ataxia telangiectasia protein acts as a crucial cell cycle checkpoint control during the repair of DNA damage [73]. In the presence of DSBs, ATM kinase phosphorylates proteins involved in DNA damage repair, cell cycle arrest, and apoptosis [74]. ATM deficiency is correlated with neuronal cell death in the human AD brain, thus potentially playing a key role in the pathogenesis of AD [75]. Reactive oxygen species can play undue stress on DNA replication and thus also result in genomic instability, Aβ accumulation, and dysregulation of the cell cycle [76]. Tumor suppressor protein p53 has also been implicated in AD as it responds to the accumulation of DSBs by promoting apoptosis and senescence [77]. Understanding of how DSBs cause neuronal damage can allow for other avenues of therapy for neurodegenerative diseases. For example, considering the role of ATM deficiency in AD progression, a pharmacological activator of ATM can be a potential therapeutic agent.

Additionally, NHEJ stimulators can help reduce the risk of neurodegenerative diseases as NHEJ activity is reduced in these diseases. Enhancing DSB repair ability can potentially promote neuronal survival and function [78]. Sirt1 stimulates activity of HDAC1, which is necessary in NHEJ for DSB repair, via deacetylation [79]. Therefore, a deficiency in Sirt1 or HDAC1 in neurons could lead to increased susceptibility to DSB formation and accumulation, suggesting that Sirt1 or HDAC1 activation can offer therapeutic effects.

In another study, Dileep et al. (2023) used both human postmortem brain samples and mouse models of neurodegeneration to determine the impact of DSBs on the genome of AD patients and found a significant association between the expression of DNA repair genes and AD pathology in AD brains. The study demonstrates that DSBs can lead to mosaic genome structural variations and disrupt 3D genome organization in neurons, contributing to the pathological process of neurodegenerative diseases. Increased mosaic gene fusions from genome structural variations were linked to increased cohesin, an important protein that organizes the 3D genome and aids in

DSB repair, DNA damage, and senescence-like gene expression in AD [80].

The clinical use of DSBs as biomarkers for neurodegenerative disease detection and progression has been limited. Kapaki et al. (2022) provides insight into novel CSF biomarkers that track autoimmune, inflammatory, and neurodegenerative aspects of CNS diseases and indirectly suggests that the role of biomarkers like DSBs can be utilized in indicating neuronal death. Elevation of such biomarkers could serve as a crucial indicator of the underlying neurodegenerative processes; this emphasizes the need for further exploration into their clinical utility [81].

Currently, there are many studies that collectively discuss the potential of using DSBs in monitoring the detection and the progression of neurological degradation from oxidative stress, mutated protein kinases and neuro-immunological disorders [82–87]. However, while the articles stress the significance of DSBs in disease progression, they stop short of detailing direct measurement methodologies thus indicating a gap in the literature regarding the clinical application of DSBs to be used as a direct diagnostic tool [82–87]. Poty et al. (2020) discusses the use of DSBs in monitoring early therapeutic responses and the effects of radiation therapy in cancer, using PET imaging with the [^{89}Zr]Zr-DFO-anti-γH2AX-TAT radiotracer for the imaging of response following the induction of DSBs [88]. While this study is primarily focused on cancer, the methodology presents an avenue that could be utilized in future research to assess the presence and severity of DSBs in clinical settings.

The current body of work highlights the potential utility of DSBs in understanding neurodegenerative diseases. Yet a significant gap in developing a precise, clinically applicable technique for DSB measurement remains. This gap represents an area for future research to enhance the present diagnostic capabilities for neurodegenerative diseases.

CONCLUSIONS AND FUTURE DIRECTIONS

The elderly population is rapidly increasing across the globe. This brings forth the pressing need to understand and address complex age-related diseases like AD. Comprehension of the physiological and pathological aspects of aging and the cellular hallmarks that are characteristic to AD are imperative to treat our aging population. It is now critically important to be able to detect AD risk to individuals early in the disease course for the most effective intervention. Utilizing the following biomarkers, cfDNA and DSBs, as potential tools for early detection and prognosis of AD would represent a significant advancement in the field of geriatric medicine. Elevated cfDNA levels are associated with dementia, cognitive decline, and physical frailty and this further points to a new promising role to identify patients at high risk for AD [16]. The link between aging, neurodegeneration, and the decline in DNA repair activity in relation to DSBs further indicates the use of these biomarkers to be used in diagnosing neurodegenerative diseases [68–71].

The implications of these findings are multifactorial regarding understanding AD and the treatment of neurodegenerative disorders. They offer a window for early intervention which is crucial in managing AD. Additionally, the ability to identify individuals with an elevated risk of developing AD could lead to more targeted and effective preventative strategies. Understanding the molecular underpinnings of AD through these biomarkers could lead to the development of novel therapeutic approaches to apply in clinical practice.

Despite these new promising findings, several challenges remain to combating neurodegenerative diseases. The search for a gold standard in healthy aging assessment and AD diagnosis is ongoing despite the promises of these biomarkers. While cfDNA and DSBs show promise, their sensitivity, specificity, and practical applicability in clinical settings still needs further validation. Ethical and psychological implications of early diagnosis in the absence of definitive curative treatments also warrant careful consideration by clinicians.

Future research into AD should focus on refining these biomarkers, exploring their integration into clinical practice, and understanding the broader implications associated with them. Additionally, there is a need for more comprehensive studies that consider the multifactorial nature of aging and AD, encompassing genetic, environmental, and lifestyle factors.

As the demographic landscape continues to shift towards an older population, the urgency to advance our understanding of aging and age-related diseases cannot be overstated. The potential of cfDNA and DSBs as biomarkers in AD offers a promising avenue of advancement. Further research is needed to understand gender dependent cfDNA and DSBs changes in the blood, CSF, and other extracellular fluids.

AUTHOR CONTRIBUTIONS

Michelle Nguyen (Data curation; Formal analysis; Investigation; Methodology); Colby Wood (Conceptualization; Data curation; Formal analysis; Investigation); Andres Rios (Data curation; Formal analysis; Investigation); Zach Salter (Data curation; Formal analysis; Methodology); Hemachandra Reddy (Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Project administration).

ACKNOWLEDGMENTS

The authors have no acknowledgments to report.

FUNDING

The research and relevant findings presented in this article was supported by the National Institutes of Health (NIH) grant, AG079264.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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